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Rapid, Simple, Quantitative, and Highly Sensitive Antibody Detection for Lyme Disease▽

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There is currently a need for improved serological tests for the diagnosis and monitoring of Lyme disease, an infection caused by *Borrelia burgdorferi*. In the present study, we evaluated luciferase immunoprecipitation systems (LIPSs) for use for profiling of the antibody responses to a panel of *B. burgdorferi* proteins for the diagnosis of Lyme disease. Initially, serum samples from a cohort of patients and controls ($n = 46$) were used for training and were profiled by the use of 15 different *B. burgdorferi* antigen constructs. For the patient sera, the antibody responses to several *B. burgdorferi* antigens, including VlsE, flagellin (FlaB), BmpA, DbpA, and DbpB, indicated that the antigens had high levels of immunoreactivity. However, the best diagnostic performance was achieved with a synthetic protein, designated VOVO, consisting of a repeated antigenic peptide sequence, VlsE-OspC-VlsE-OspC, Analysis of an independent set of serum samples ($n = 139$) used for validation showed that the VOVO LIPS test had 98% sensitivity (95% confidence interval [CI], 93% to 100%; $P < 0.0001$) and 100% specificity (95% CI, 94% to 100%; $P < 0.0001$). Similarly, the C6 peptide enzyme-linked immunosorbent assay (ELISA) also had 98% sensitivity (95% CI, 93% to 100%; $P < 0.0001$) and 98% specificity (95% CI, 90% to 100%; $P < 0.0001$). Receiver operating characteristic analysis revealed that the rates of detection of Lyme disease by the LIPS test and the C6 ELISA were not statistically different. However, the VOVO LIPS test displayed a wide dynamic range of antibody detection spanning over 10,000-fold without the need for serum dilution. These results suggest that screening by the LIPS test with VOVO and other *B. burgdorferi* antigens offers an efficient quantitative approach for evaluation of the antibody responses in patients with Lyme disease.

Lyme disease is caused by the spirochete *Borrelia burgdorferi*, which is transmitted by the bite of a deer tick (*Ixodes* sp.) (24, 29). One of the first signs of *B. burgdorferi* infection is erythema migrans (EM), a skin lesion that appears within a few days at the site of the bite. Subsequently, the spirochetes can disseminate into the bloodstream and then to various target tissues and cause neurological, cardiac, and rheumatological complications (24, 29). Some individuals develop post-Lyme disease syndrome (PLDS) and have lingering symptoms, such as fatigue, musculoskeletal pain, and cognitive impairment (22, 24, 29).

Currently, the Centers for Diseases Control and Prevention (CDC) recommends the use of a two-tier approach for serological testing for Lyme disease (1). The two-tier approach includes an initial enzyme immunoassay or immunofluorescence assay, followed by Western blotting for positive or borderline samples. The limitations of the two-tier testing approach include a low sensitivity in the very early stages of the *B. burgdorferi* infection, subjectivity in the interpretation of the Western blot bands, and the significant amount of time and the significant cost for the process. Moreover, current antibody tests do not distinguish between active and prior infection. Therefore, there is a need for sensitive and specific tests for the identification and monitoring of individuals with Lyme disease.

Several tests, which employ recombinant spirochetal proteins, have shown promising results (15, 17, 21). A simple enzyme-linked immunosorbent assay (ELISA) with the C6 peptide, a 26-mer synthetic peptide analogue of the invariable region 6 (IR6) of the VlsE variable major protein-like sequence has been shown to be highly sensitive and specific for the detection of *B. burgdorferi* infection (2, 14, 19, 20). While there are intriguing data on the use of the level of antibody against C6 to monitor the response to antibiotic therapy in patients with Lyme disease (16, 18, 26, 27), those studies are hampered by the limited dynamic range of solid-phase immunoassays and the need to perform time-consuming and cumbersome serum dilutions to obtain values in the linear range. A test capable of monitoring the response to antibiotic therapy and distinguishing between active and prior infection would be a major advance in the field.

Luciferase immunoprecipitation systems (LIPSs) provide a powerful new approach to serological testing for antibodies associated with many different human pathogens (4). The LIPS is based on the fusion of protein antigens to a light-emitting enzyme reporter, *Renilla* luciferase (Ruc), and then the use of these antigen fusions in immunoprecipitation assays with serum samples and protein A/G beads. After the beads are washed, the level of light production is measured, yielding highly quantitative antibody titers. Due to the liquid-phase nature of the LIPS assay and the highly linear light output of

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the luciferase reporter, some antibodies can be detected without serum dilution over a dynamic range of detection often spanning 7 orders of magnitude. While the LIPS test has already been shown to have a high degree of sensitivity for the detection of fungal (5), helminthic (28), filarial (10, 12), and a variety of viral (3, 5–9, 11) infectious agents, its utility for the accurate evaluation of humoral responses to bacterial pathogen antigens has not been assessed. In this report, we describe the initial development and evaluation of LIPS tests for the serological diagnosis of Lyme disease.

MATERIALS AND METHODS

Patient sera. Serum samples were obtained from patients and volunteers under institutional review board-approved protocols at the National Institute of Allergy and Infectious Diseases, NIH. The serum samples ($n = 46$) in the initial training set were from 8 healthy volunteer (HV) controls and 38 patients with various manifestations of Lyme disease. The 38 samples with potential manifestations of Lyme disease were from 11 patients with EM, 8 patients with multiple erythema migrans (MEM), 6 patients with Lyme arthritis, 2 patients with early neuroborreliosis, 1 patient with late Lyme neuroborreliosis, and 10 patients with PLDS.

The cohort of serum samples used for validation consisted of samples from 84 patients with Lyme disease and 55 controls. The control samples included 15 samples from HVs; 15 antinuclear antibody (ANA)-positive samples; 12 rheumatoid factor (RF)-positive samples; 3 rapid plasma reagin (RPR) test-positive samples; 3 samples from patients with southern tick-associated rash illness (STARI); 2 samples from patients with spotted fever rickettsial infections; and 1 sample each from patients with Behcet's disease, human monocytic ehrlichiosis, hematoma, a tick bite, and multiple sclerosis. The 84 samples from patients with Lyme disease originated from 80 separate patients. There were 22 samples from patients with EM, 13 samples from patients with MEM, 16 samples from patients with acute neuroborreliosis, 3 samples from patients with cardiac involvement, 12 samples from patients with Lyme arthritis, 8 samples from patients with late neuroborreliosis, and 10 samples from patients with PLDS. More than one serum sample from four patients was tested: one patient with two episodes of EM, one patient with EM who later developed late neuroborreliosis, and two patients with MEM who developed PLDS. Of the 22 Lyme disease patients with EM, culture and PCR of skin biopsy specimens was performed for only 5 patients: 4 were PCR positive and 3 were culture positive, and for 1 patient the culture was contaminated. Of the 80 samples from patients with Lyme disease, 3 patients had acquired the disease in Europe. The codes for the validation cohort were broken only after the titers were established and the categorization had been made. The antibody titer results for the validation cohort obtained by the LIPS test were also compared with those determined by the C6 ELISA (Immunetics Inc., Boston, MA). The samples were also tested by using the two-tier algorithm at Mayo Medical Laboratories. Patients with late manifestations of Lyme disease were positive by immunoblotting by the use of established criteria (1).

Generation of *B. burgdorferi* luciferase recombinant proteins. pREN2, a mammalian Ruc expression vector, was used to generate all plasmids. The *B. burgdorferi* genes were amplified by PCR with specific linker-primer adapters and synthetic cDNA templates assembled in the laboratory of one of the investigators or were obtained from Blue Heron Biotechnology (Seattle, WA). Gene-specific primers were then used in the PCR amplifications to generate cDNA sequences for cloning of the C-terminal fusions of Ruc. For each C-terminal fusion, a stop codon was included at the end of the coding sequence. Several of the antigens, including FlaB, BmpA, OspC, BBK32, and CRASP-2, were derived from the protein gene products of *B. burgdorferi* strain 31, while two other antigens, DbpA and DbpB, were from *B. burgdorferi* sensu lato and *B. burgdorferi* strain Zs7, respectively. The nucleotide and protein sequences for these and other *B. burgdorferi* proteins tested are available upon request. The sequences of two constructs, VlsE-Δ1 and VlsE-Δ2, contained peptide sequences from the C terminus of VlsE of *B. burgdorferi* strain 31. The VlsE-Δ1 protein sequence contained 56 amino acids from VslE and included all but the 4 C-terminal amino acids from the IR6 peptide. The VlsE-Δ2 protein contained the last 160 amino acids of the C terminus of VlsE and included the internal IR6 peptide (25 amino acids). In addition, the nucleotide and protein sequence for VOVO, a hybrid molecule, has GenBank accession number GU134803. The peptide encoded by VOVO is MKKDDQIAAAIALRGMAKDGKFAVKELTS*PVVAESKKP*MKKDDQIAAAMVLRGMAK-DGQFALK*PVVAESPKKP*, in which the two peptides sequences from two different VlsE peptides (derived from *B. burgdorferi* strain 31 and *B. garinii* IP90) are underlined and the OspC sequences (from *B. burgdorferi* strain 31) are in italics. DNA sequencing was used to confirm the integrity of all the DNA constructs. Plasmid DNA was then prepared from the different pREN2 expression vectors by using a Qiagen midipreparation kit (Valencia, CA).

LIPS analysis. Following transfection of the mammalian expression vectors, crude protein extracts were obtained, as described previously (4). A detailed protocol for the LIPS assay is now available, along with a corresponding technical video, from the *Journal of Visualized Experiments* (http://www.jove.com/index/details.stp?ID=1549) (4). In this high-throughput antibody testing system, serum, buffer, and Ruc-antigen extracts are incubated in a microtiter plate for 60 min at room temperature on a rotary shaker. Next, 5 μl of a 30% suspension of Ultralink protein A/G beads (Pierce Biotechnology, Rockford, IL) in phosphate-buffered saline (PBS) was added to the bottom of each well of a 96-well filter high-throughput system plate (Millipore, Bedford, MA). The antigen-antibody reaction mixture was then transferred from the microtiter plate to the filter plate, and the mixture was incubated for an additional 60 min at room temperature on a rotary shaker. In this step, the protein A/G beads mainly capture IgG antibodies and poorly retain IgA and IgM (13). Next, the filter plates containing the protein A/G beads and the antigen-antibody mixture were washed extensively with buffer. The number of light units (LUs) on this filter plate was then measured in a Berthold LB 960 Centro microplate luminometer (Berthold Technologies, Bad Wilbad, Germany) with a coelenterazine substrate mix (Promega, Madison, WI). The data presented in Fig. 1 are $\log_{10}$-transformed values, which are coded by the use of a color palette ranging from red to green, indicating high and low titers, respectively. All LU data for VOVO with the samples from the validation cohort were obtained from the averages of two separate experiments and were corrected for the background by subtracting the LU values for the beads alone.

Statistical analysis. Statistical analysis was performed with GraphPad Prism software (San Diego, CA). Due to the wide dynamic range of the LIPS test data, the results for the quantitative antibody levels for the control and Lyme disease patient serum samples are reported as the geometric mean titer (GMT) ± 95% confidence interval (CI). The correlation of the antibody responses from the $\log_{10}$-transformed LIPS test values with the C6 ELISA values was assessed by the Spearman rank test (r_S). For determination of the cutoff limits for each of the LIPS tests, the mean value of the control samples plus 5 standard deviations (SDs) was used and is indicated by the long solid lines in Fig. 2 and 3. The performance of the VOVO LIPS test and ELISA was evaluated by using the area under the curve (AUC) from receiver operator characteristic (ROC) analysis.

Nucleotide sequence accession numbers. The nucleotide sequences of VlsE-Δ1 and VlsE-Δ2 have been deposited in GenBank under accession numbers GU182319 and GU182320, respectively.

RESULTS

LIPS assay detection of antibody responses to a panel of *B. burgdorferi* antigens. Previous studies at various laboratories have identified a large number of antigens useful for serological screening for *B. burgdorferi* infection. Fifteen different *B. burgdorferi* antigen constructs, including flagellin (FlaB), BmpA, DbpA, DbpB, OspC, and two different VlsE constructs, were initially assembled synthetically and constructed as C-terminal fusions with Ruc. Evaluation of these different antigens by the LIPS test began by testing a small cohort of serum samples ($n = 46$) consisting of serum samples from 8 HV controls and 38 patients with various manifestations of Lyme disease. The different immunoreactivities to this antigen panel were visualized by using a heat map to graphically display the antibody responses by using a $\log_{10}$ scale for the most informative antigens (Fig. 1). Other *B. burgdorferi* antigens (including BBK32; CRASP-2; OspA; and several OspC, DbpA and DbpB protein constructs containing their signal sequences) showed weak signals or poor sensitivities (data not shown). Deletion of the signal peptide from some of these proteins (e.g., DbpA and DbpB) improved the performance. On the basis of the mean plus 5 standard deviations of the controls, the most sensitive and specific antigen in the initial

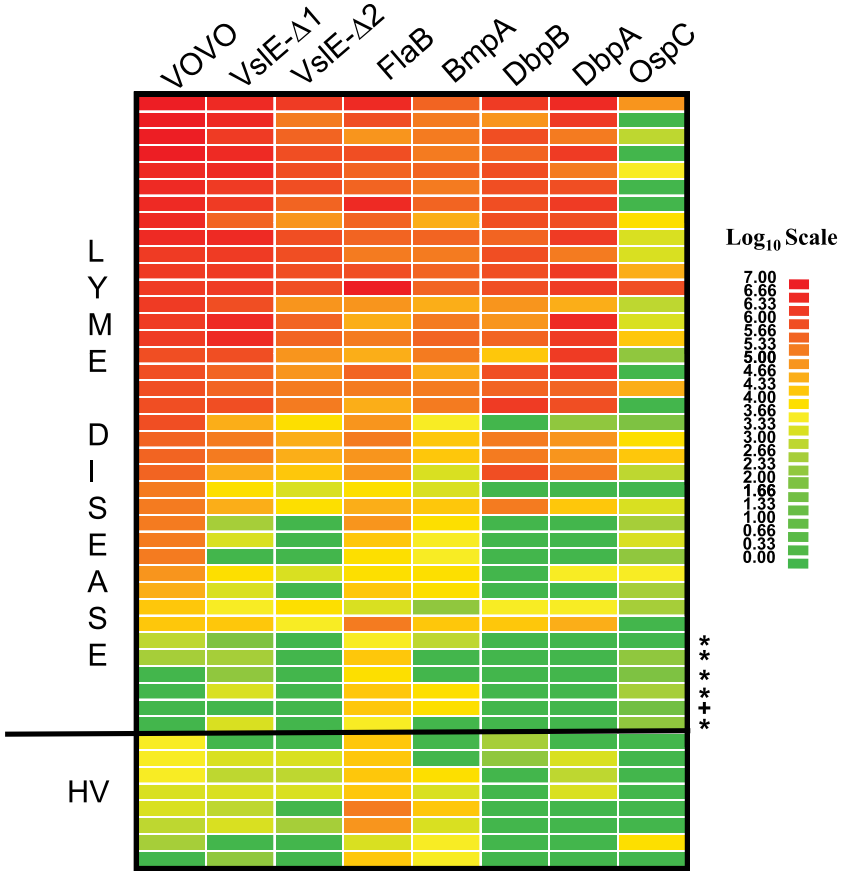

FIG. 1. Heat map representation of patient antibody responses to VOVO and seven other *B. burgdorferi* antigens. The antibody titer values for each serum sample were $\log_{10}$ transformed and then color coded, as indicated by the $\log_{10}$ scale to the right of the heat map, in which the signal intensities range from red to green, indicating high and low titers, respectively. Each row represents the results for one serum sample tested with the different antigens. The results for the most informative antigen, VOVO, are shown on the far left. Samples from five patients with EM (∗) and one patient with PLDS (+) considered negative by the assay with VOVO are indicated to the right of the heat map.

panel was VlsE-Δ2, followed by VlsE-Δ1. Two of the antigens, DbpA and DbpB, showed responses similar to each other but were less sensitive than either of the VlsE fusions (Fig. 1).

Due to the less than optimal performance from the two different VlsE constructs in the LIPS assay, an additional VlsE protein construct was designed and tested. This new antigen, designated VOVO, was a synthetic recombinant protein containing two alternating copies of immunoreactive peptides derived from the IR6 region of VlsE and the conserved C-terminal region of OspC (23). Two slightly different VlsE peptide sequences were used and were derived from strains *B. burgdorferi* B31 and *B. garinii* IP90. The rationale behind the design of VOVO was that the repeated antigenic peptides from different immunodominant epitopes might detect more divergent strains, increase the sensitivity through cooperative binding, and/or expose more conformational epitopes, thereby capturing low-affinity and/or low-titer antibodies. LIPS testing analysis with VOVO showed that it was the most useful antigen of the panel and was far superior to the two VlsE constructs described above. As shown in Fig. 2, the GMT of the anti-VOVO antibody titer in the 38 samples from patients with Lyme disease was 106,400 LUs (95% CI, 22,990 to 492,700 LUs), which was markedly higher than the GMT of 559 LUs (95% CI, 62 to 5,000 LUs) for the controls (Mann-Whitney *U*

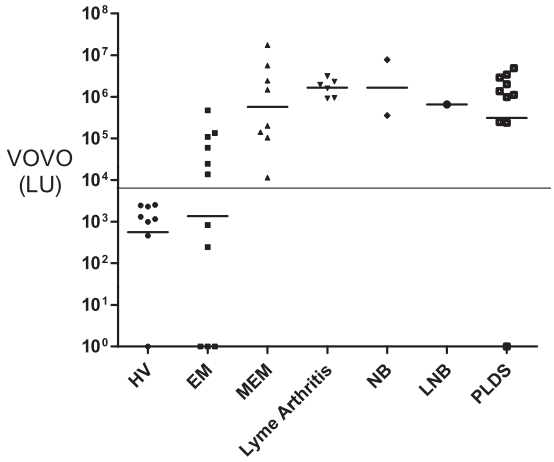

FIG. 2. Performance of the VOVO LIPS test with the pilot study serum sample set. The results obtained with serum samples from 11 patients with EM, 8 patients with MEM, 6 patients with Lyme arthritis, 2 patients with early neuroborreliosis (NB), 1 patient with late neuroborreliosis (LNB), 10 patients with PLDS, and 8 HV controls are shown. Each symbol represents a serum sample from an individual patient. Due to the wide dynamic range of the antibody titer values and the small sample size, only the geometric mean titer for each subgroup is shown (bars). The cutoff, based on the mean plus 5 SDs of the HV, is shown by the long solid line, and the number of LUs for each sample is shown on the *y* axis.

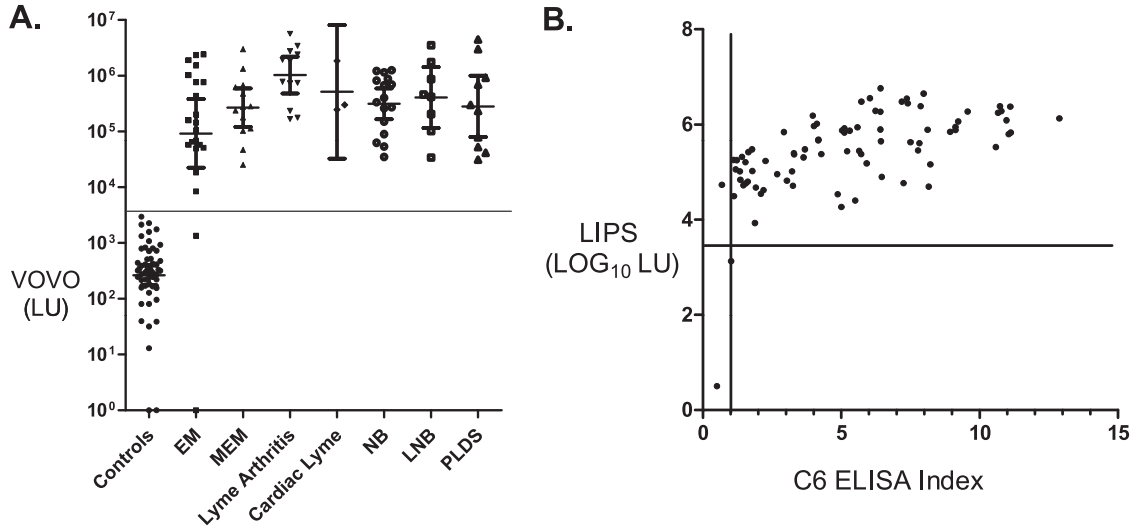

FIG. 3. The VOVO antigen has a high sensitivity and a high specificity for the detection of antibodies against *B. burgdorferi*. (A) Results for 84 serum samples from patients with Lyme disease and 55 controls. Each symbol represents a serum sample from an individual patient. The short solid horizontal lines indicate the GMT of the anti-VOVO antibody per group, and the vertical lines show the 95% confidence intervals. The cutoff based on the mean plus 5 SDs of the HV is shown by the long solid line. (B) Antibodies detected by the C6 ELISA versus the VOVO LIPS test for patients with Lyme disease. The calculated Spearman rank correlation (r_s) was 0.65 ($P < 0.0001$). The horizontal and vertical lines represent the cutoffs for the LIPS assay and the C6 ELISA index, respectively. The number of LUs for each sample is shown on the *y* axis.

test, $P < 0.0017$). By using a cutoff derived from the mean plus 5 SDs of the controls, 84% of the samples from patients with Lyme disease were seropositive for VOVO, and all samples from the uninfected controls were negative (Fig. 2). The serum samples from a patient with PLDS and five patients with EM were negative for anti-VOVO antibodies. These six samples were also negative by the C6 ELISA and a whole-cell-lysate ELISA. In the case of the EM samples, four of the five patients with EM presented within the first week of illness. These promising results suggest that VOVO might be a highly useful antigen for use in the LIPS test for the detection of humoral responses to *B. burgdorferi* infection.

Strong diagnostic performance of VOVO LIPS test with a new cohort of independent samples for validation. To test the effectiveness of VOVO and to compare the results of the VOVO LIPS test with those of the C6 ELISA, a new cohort of 139 blinded serum samples used for validation was evaluated. Similar to the results obtained with the training set, the mean anti-VOVO antibody titer in the 84 samples from patients with Lyme disease was 272,000 LUs (95% CI, 171,900 to 430,200 LUs), which was 1,038-fold higher than the antibody titer of 262 LUs (95% CI, 174 to 397 LUs) for the 55 controls (Mann-Whitney U test, $P < 0.0001$). In order to determine the sensitivity and specificity, a diagnostic cutoff value of LUs on the basis of the mean plus 5 SDs of the control samples (3,553 LUs) was used. By use of this cutoff, the VOVO LIPS test showed a 98% sensitivity (82/84 Lyme samples; 95% CI, 93% to 100%, $P < 0.0001$) and a 100% specificity (55/55; 95% CI, 94% to 100%) for the validation cohort (Fig. 3A). Similarly, the C6 ELISA had a 98% sensitivity (82/84 Lyme samples; 95% CI, 93% to 100%, $P < 0.0001$) and a 98% specificity (54/55; 95% CI, 90% to 100%). The three patients who acquired Lyme disease in Europe were positive by both the C6 ELISA and the VOVO LIPS test. Unlike the limited dynamic range of the C6 ELISA (0.11 to 12.89 optical density units), the VOVO LIPS test showed a markedly greater dynamic range spanning over 10,000-fold (Fig. 3A) and did not require additional serum dilution. Lastly, correlation of the $\log_{10}$-transformed LIPS test values with the C6 ELISA index values for the samples from patients with Lyme disease showed that the results of the two assays tracked each other ($r_s = 0.65$, $P < 0.0001$).

DISCUSSION

This study demonstrates the ability of the LIPS test to robustly detect the titers of antibodies to a panel of *B. burgdorferi* antigens with high degrees of diagnostic sensitivity and specificity. In the LIPS test, recombinant proteins are produced in mammalian cells and are directly tagged with the highly sensitive Ruc reporter enzyme. The most effective antigen in the LIPS test format was the synthetically designed VOVO protein, which distinguished 98% of the samples from patients with Lyme disease as being positive and with 100% sensitivity with samples from the validation sample cohort. Similarly, the C6 ELISA, which uses a chemically synthesized peptide immobilized on the wells of microtiter plates, had 95% sensitivity and 98% specificity. One of the key advantages of the VOVO LIPS test is the large dynamic range of detection of antibodies because of the solution-phase assay format and the high linear output of the Ruc reporter. The ability of the LIPS test to detect sensitive and robust antibody titers may have other applications, especially serial testing and assessment of the antibody response after antibiotic therapy. Moreover, the ease and simplicity of the LIPS assay allow it to be used to test thousands of samples for the presence of *B. burgdorferi* antibodies in a high-throughput format.

The detection of low-affinity antibodies can often pose a diagnostic challenge. One approach to increase the affinity of

an antibody for a target involves the use of antigen clustering to enhance antibody binding. For example, a streptavidin-biotin dimerization approach was used to generate antigen tetramers, which showed enhanced autoantibody detection (25). An even simpler approach which involves the use of different repeated immunodominant peptides as a single synthetic fusion protein is described here, specifically, VOVO, a recombinant protein containing repeated immunodominant peptides of two different peptides from VlsE and two repeated peptides from OspC. This general approach of employing repeated peptides as a single recombinant protein in the LIPS system may be useful for the development of antibody-based tests for the detection of other antigens, including characterized immunodominant epitopes and immunoreactive peptides identified from phage display (30).

Despite the strong diagnostic performance of VOVO LIPS test, the results presented here are still preliminary. For clinical testing, larger cohorts are needed to further standardize the VOVO LIPS assay and establish the exact cutoff needed to detect the borderline-positive samples. It is likely that the incorporation of additional VlsE C6 peptide sequences from other species, such as *B. afzelii*, as well as other *B. burgdorferi* strains, might further increase the sensitivity of the assay, especially when samples from patients with Lyme disease from Europe and other diverse locations are analyzed. The other *B. burgdorferi* antigens identified in the initial small training set, which were not extensively studied here, may also be highly informative in other studies. LIPS assay detection of patient-specific antibody responses to some of the *B. burgdorferi* antigens might have additional utility for stratifying patient populations on the basis of their clinical symptoms, duration of infection, and response to drug therapy.

In summary, the VOVO LIPS assay shows extraordinary potential as a high-throughput screening tool for identifying antibodies against *B. burgdorferi.* It will be of interest to determine whether this VOVO LIPS test is useful for monitoring antibody titer changes over time in longitudinal samples from patients after antibiotic therapy for Lyme disease.

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Three of the authors (P.D.B., M.J.I., and A.M.) have submitted a patent application for the LIPS test for the detection of anti-*B. burgdorferi* antibodies.

Informed consent was obtained from all patients, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services under several NIAID Institutional Review Board-approved protocols.

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